AD NUMBER

AD821491

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; AUG 1967. Other requests shall be referred to Department of the Army, Fort Detrick, MD 21701.

AUTHORITY

SMUFD D/A ltr, 29 Sep 1971

TECHNICAL MANUSCRIPT 409

A HEAT-STABLE CHEMICALLY DEFINED MEDIUM FOR GROWTH OF ANIMAL CELLS IN SUSPENSION

Stanley C. Nagle, Jr.

AUGUST 1967

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

DDC AVAILABILITY NOTICES

Qualified requesters may obtain copies of this publication from DDC.

Foreign announcement and dissemination of this publication by DDC is not authorized.

Release or announcement to the public is not authorized.

DISPOSITION INSTRUCTIONS

Destroy this publication when it is no longer needed. Do not return it to the originator.

The findings in this publication are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701

TECHNICAL MANUSCRIPT 409

A HEAT-STABLE CHEMICALLY DEFINED MEDIUM FOR GROWTH OF ANIMAL CELLS IN SUSPENSION

Stanley C. Nagle, Jr.

Medical Bacteriology Division
BIOLOGICAL SCIENCES LABORATORY

Project 1C522301A082

August 1967

ABSTRACT

Procedures are described for preparation of heat-stable chemically defined medium for the cultivation of animal cells in suspension. Growth of cat kidney, HeLa, and L cells of 34.1, 33.8, and 47.1 x 10^5 cells per ml was recorded after 6, 10, and 10 days of incubation, respectively.

I. INTRODUCTION

The advantages of a chemically defined medium in tissue culture have been recognized for several years. A chemically defined medium that is also heat-stable has additional advantages: processing in a single vessel requires less handling, and thereby reduces chance of contamination, availability of autoclaves in most laboratories eliminates the necessity for filtration equipment, and industrial sterilizing equipment may be employed for large quantities.

A filter-sterilized, chemically defined medium was described in 1963 that supported growth in suspension of several cell lines;[1] the medium was later simplified by omission of insulin.[2] A heat-stable serum-free medium containing peptone was described in 1966.[3] The present paper describes the development of a heat-stable, chemically defined medium that supports growth of animal cells in suspension culture comparable to growth obtained in filter-sterilized media.

II. MATERIALS AND METHODS

A. MEDIA

Except for methylcellulose, glutamine, and sodium bicarbonate, all ingredients to make 1,000 ml of the chemically defined medium (Table 1) were dissolved in 937.5 ml of distilled water, placed in 8-oz or 16-oz prescription bottles (187.5 ml or 375 ml), and autoclaved with loose caps at 121 C for 15 minutes. Methylcellulose was prepared by autoclaving at 121 C for 15 minutes as a 2% solution in 8-oz prescription bottles. The 5% sodium bicarbonate solutions were autoclaved in nearly filled, tightly stoppered serum bottles of various sizes. Glutamine was autoclaved as a dry powder (180 mg, sufficient for 400 ml of complete medium) in a stoppered 15-ml serum bottle for 1 hour at 121 C. Each of these components was stored at 5 C. Serum bottles were fitted with rubber serum-bottle stoppers and sealed with metal closures. Antibiotics (dissolved in sterile distilled water as 200X solutions and stored at -25 C) were added to most experimental media at concentrations of 100 μg of streptomycin per ml and 100 units of penicillin per ml. In experiments testing the addition of filter-sterilized medium components, a 0.22-μ membrane filter* was used. Components were combined to prepare 1X medium as follows: to 375 ml of incomplete medium were added 20 ml of 2% methylcellulose, 2 ml of 200X antibiotics (when used), and 3 ml of 5% sodium bicarbonate for partial neutralization. The autoclaved glutamine powder also was added aseptically at this time, either by removing the metal closure and rubber stopper and dumping the 180 mg of dry powder into

* Millipore.

TABLE 1. HEAT-STABLE CHEMICALLY DEFINED MEDIUM

Component	Concentration, mg/liter	Component	Concentration, mg/liter
Amino acids		Salts, etc.	
L-Arginine·HCl	100	NaCl	7,400
L-Cysteine·HCl	75	KCl	400
L-Histidine·HCl	60	$NaH_2PO_4 \cdot H_2O$	100
L-Isoleucine	150	$MgCl_2 \cdot 6H_2O$	275
L-Leucine	300	$CaCl_2 \cdot 2H_2O$	265
L-Lysine·HCl	300	Phenol red	10
L-Methionine	60	Carbon sources	
L-Phenylalanine	120	Glucose	1,000
L-Threonine	135	Na pyruvate	100
L-Tryptophan	60	Vitamins[d]	
L-Tyrosine	120	D-Biotin	1.0
L-Valine	150	Choline Cl	1.0
Aseptic additions		Folic acid	1.0
Methylcellulose, 15 cps	1,000[a]	Niacinamide	1.0
L-Glutamine	450[b]	Ca pantothenate	2.0
$NaHCO_3$	500[c]	Pyridoxal·HCl	1.0
		Thiamine·HCl	1.0
		i-Inositol	1.0
		Riboflavin	0.1
		B_{12}	0.002

a. Added as 2% solution.
b. Added as powder.
c. Added as 5% solution.
d. Added as 100X solution.

the medium, or by using a syringe containing 5 to 10 ml of medium to dissolve the glutamine and then adding this solution to the remaining medium. The completed medium was stored at 5 C. An additional 0.5 to 1.0 ml of bicarbonate solution was added to each 25 ml of medium contained in the growth bottles at time of inoculation with cells. This step assured an initial pH of approximately 7.2 to 7.4.

B. CELL LINES

Mouse fibroblast (L), HeLa, and cat kidney (CK) cells that had been growing in suspension continuously for more than 3 years in the filter-sterilized chemically defined medium were employed in these studies. No pleuropneumonia-like organisms (PPLO) were detectable in these cell cultures upon testing on standard PPLO medium.

C. INCUBATION AND CELL ENUMERATION

Growth of suspension cultures was initiated from populations of 1 to 3 x 10^5 cells per ml. Cultures were incubated at 34 to 36 C in rubber-stoppered 100-ml serum bottles containing 25 ml of medium on a New Brunswick Gyrotory shaker operating at 124 to 130 rpm. At appropriate intervals, numbers of viable cells were determined in the hemocytometer by the trypan blue procedure of McLimans et al.[4] Media were changed by centrifuging the serum bottle cultures at 1,000 rpm for 3 to 5 minutes, decanting the supernatant, and replacing it with fresh medium.

III. RESULTS

Table 2 shows the results of an experiment with L cells in which separate groups of filter-sterilized components were tested in an autoclaved basal mixture. Two cycles of growth were carried out in these media to insure the exhaustion of any possible cellular pool of potentially heat-labile medium components; after one 7-day period of growth all cultures were adjusted to about 3 x 10^5 cells per ml and reincubated. Media were changed daily. Results of the second growth cycle, shown in Table 2, indicated that glutamine was the most heat-labile medium component and that growth occurred if glutamine was sterilized by filtration or by autoclaving the dry powder.

Growth curves for CK, HeLa, and L cells were determined in the autoclaved medium containing dry-sterilized glutamine; these results are shown in Figure 1. Yields of 34.1, 33.8, and 47.1 x 10^5 cells per ml were recorded for CK, HeLa, and L cells after 6, 10, and 10 days of incubation, respectively. These cell lines had been cultured continuously for almost a year in the autoclaved defined medium (subsequent to growth in the filter-sterilized medium) prior to growth curve measurements.

TABLE 2. GROWTH OF L CELLS IN SUSPENSION IN HEAT-STERILIZED DEFINED MEDIA CONTAINING FILTER- OR HEAT-STERILIZED COMPONENTS

Sterilization Method	Growth in 7 days[a], x 10^5/ml
Autoclaved complete medium[b]	6.9
Autoclaved complete medium except glucose[c]	5.6
Autoclaved complete medium except pyruvate[c]	8.2
Autoclaved complete medium except vitamins[c]	4.9
Autoclaved complete medium except amino acids[c] (excluding glutamine)	5.5
Autoclaved complete medium except glutamine[c]	20.8
Autoclaved complete medium except dry-autoclaved glutamine	21.3
Filtration of complete medium[b]	15.3

a. Second 7-day cycle of growth in respective media. 0-hour population in every case was approximately 3 x 10^5 per ml.
b. Complete except for methylcellulose, bicarbonate, and antibiotics.
c. Indicated substances were sterilized by filtration.

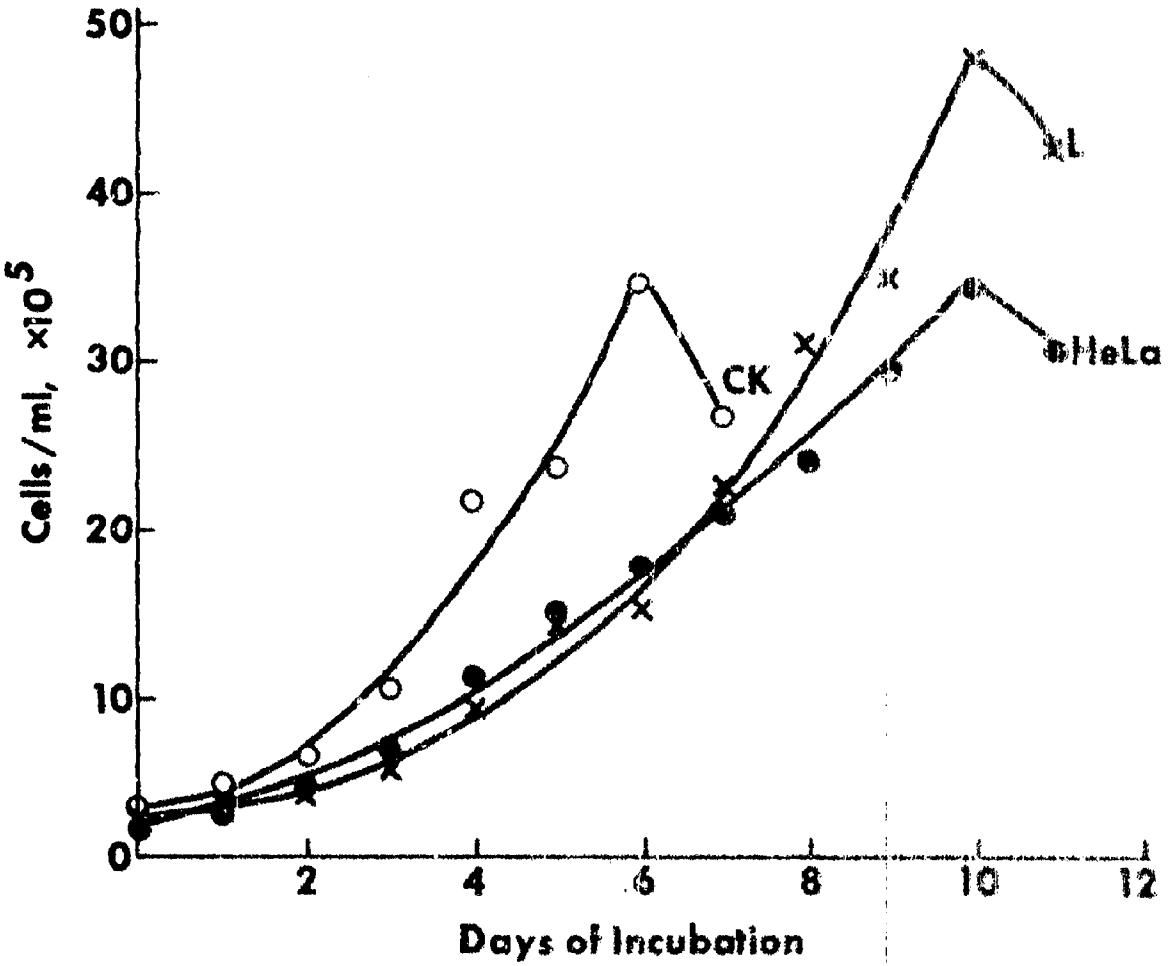

Figure 1. Growth of Animal Cells in Heat-Sterilized Chemically Defined Medium.

IV. DISCUSSION

Studies for the development of heat-stable defined media were undertaken following success with the autoclavable peptone medium. Preliminary results with L cells indicated that the lability of glutamine was responsible for loss of growth-promoting activity in the defined medium during autoclaving. Subsequent experiments showed that glutamine solutions autoclaved separately were also unsatisfactory, but that exclusion of moisture and autoclaving for as long as 8 hours gave a sterile, intact product. A routine autoclaving time of 1 hour was adopted to insure sterility of the dry product without requiring excessive time. Glutamine subjected to dry heat (170 C) for 1 hour caramelized.

Sterilization of bicarbonate solution by autoclaving was satisfactory by the method described in stoppered serum bottles. Apparently, CO_2 loss was minimal by this method because adequate buffering was possible with this material. Alternatively, solutions that are autoclaved with loose caps may be used if CO_2 is replaced by bubbling sterile gas into the autoclaved solution prior to use.

Because other medium components are recognized to be heat-labile in various degrees (e.g., vitamins, glucose), especially when combined, the possibility was considered of an unidentified contaminant organism that supplied heat-labile components to the cells. Repeated attempts to detect such organisms have not been successful.

Sergeant and Smith[8] found that a heated solution of glucose and phosphate stimulated the survival, early cell division, and attachment to glass of HeLa cells. Presumably, then, harmful effects of heat-sterilization (loss of vitamins, etc.) may be offset in some instances by the production of complex substances that are useful to the cells. Such a possibility requires testing in our system.

The medium appears to be sufficiently heat-stable when prepared as described to allow continued growth of the three cell lines tested. No diminution of growth has been noted in the past year. Cells that had been maintained in the filter-sterilized medium required no period of adaptation in the autoclaved medium for excellent growth. The medium may prove to be most useful in cell and virus research, particularly in view of the recent findings of toxic substances in certain membrane filters[9] that are used for filter-sterilization of tissue culture media.

LITERATURE CITED

1. Nagle, S.C., Jr.; Tribble, H.R., Jr.; Anderson, R.E.; Gary, N.D. 1963. A chemically defined medium for the growth of animal cells in suspension. Proc. Soc. Exp. Biol. Med. 112:340-344.

2. Nagle, S.C., Jr. 1963. Growth of animal cells in a chemically defined suspension system. Bacteriol. Proc. p. 134.

3. Nagle, S.C., Jr. 1966. A serum-free autoclaved medium for growth of animal cells in suspension. Abstracts 17th Annual Meeting of Tissue Culture Association, p. 111.

4. McLimans, W.F., Davis, B.V.; Glover, F.L.; Rake, G.W. 1957. The submerged culture of mammalian cells: The spinner culture. J. Immunol. 79:428-433.

5. Sergeant, P.P.; Smith, S. 1960. A growth-stimulating factor for an epithelial cell line in a reduced serum medium. Science 131:606-607.

6. Cahn, R.D. 1967. Detergents in membrane filters. Science 155: 195-196.

Unclassified
Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)

Department of the Army
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION

Unclassified

2b. GROUP

3. REPORT TITLE

A HEAT-STABLE CHEMICALLY DEFINED MEDIUM FOR GROWTH OF ANIMAL CELLS IN SUSPENSION

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)

Eagle, Stanley C., Jr.

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
August 1967	12	6

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1C522301A082	Technical Manuscript 409
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT

Qualified requesters may obtain copies of this publication from DDC.
Foreign announcement and dissemination of this publication by DDC is not authorized.
Release or announcement to the public is not authorized.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Department of the Army Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

Procedures are described for preparation of a heat-stable chemically defined medium for the cultivation of animal cells in suspension. Growth of cat kidney, HeLa, and L cells of 34.1, 33.8, and 47.1 x 10^5 cells per ml was recorded after 6, 10, and 10 days of incubation, respectively.

14. Key Words

*Cells
*Suspension
*Media
Tissue culture
HeLa
L cells
Kidney

DD FORM 1 NOV 68 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

Unclassified
Security Classification